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***In vivo* and *In vitro* Chondrocyte Metabolism in
Relationship to the Development of Tibial
Dyschondroplasia in Broiler Chickens**

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Title

In Vivo and In Vitro Chondrocyte Metabolism in Relationship to the
Development of Tibial Dyschondroplasia in Broiler Chickens

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Final Report

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Abstract

Skeletal deformities are a significant financial and welfare problem for the world poultry industry. Tibial dyschondroplasia (TD) is the most prevalent skeletal abnormality found in young broilers, turkeys and ducks. Tibial dyschondroplasia results from a perturbation of the sequence of events in the epiphyseal growth plate, the tissue responsible for longitudinal bone growth. The purpose of this investigation was to test the hypothesis that TD was the result of a failure of growth plate chondrocytes to differentiate and express the chemotactic molecules required for cartilage vascularization.

In this investigation *in situ* hybridization and immunocytochemical techniques were used to study chondrocyte gene products associated with cartilage maturation and vascularization such as osteopontin, osteonectin, type X collagen, and alkaline phosphatase. All markers were present in the growth plate tissue anterior to the TD lesion but were greatly diminished in the TD lesion. Thus, rather than not acquiring the markers for hypertrophy, it appears that the growth plate chondrocytes reach a certain stage of hypertrophy and then de-differentiate into cells which resemble chondrocytes in the pre-hypertrophic zone. Similar patterns were observed in all TD tissues examined whether the lesions were spontaneous or induced by dietary treatments or genetic selection. The decrease in gene expression can at least be partially explained by the fact that many of the dysplastic chondrocytes show classic signs of apoptosis. These results provide an explanation for the observation that a variety of genes show reduced expression in the TD lesion when examined by *in situ* hybridization. This would suggest that future research should focus on the earliest detectable stages in the development of TD and examine endocrine and autocrine factors which cause chondrocytes to de-differentiate and undergo premature apoptosis.

OBJECTIVES OF ORIGINAL PROPOSAL

This project was developed to study metabolism of chondrocytes in normal growth plate and tibial dyschondroplastic tissue. The morphology of tibial dyschondroplasia is shown in figure 1. The basis of this proposal was the fact that there were differences in staining properties of the normal and TD tissue. These differences are shown in figure 2. Thus, it was hypothesized that the chondrocytes in the TD tissue failed to synthesize the gene products associated with the normal progression of chondrocytes to the hypertrophic state such as that shown in figure 3. This led to the development of the following objectives to test the above hypothesis.

Objective 1. Compare the *in vitro* response of normal and dysplastic chondrocytes to endocrine, paracrine and autocrine factors thought to control the life cycle of epiphyseal growth plate chondrocytes.

Objective 2. Determine the gene products of normal and dyschondroplastic chondrocytes with special emphasis on those extracellular matrix components (ECM) components specifically associated with cell hypertrophy and the formation of a calcified cartilage matrix.

Objective 3. Utilize histochemical and immunocytochemical techniques to study the formation and distribution of extracellular matrix components in normal and dysplastic tissue.

RESULTS

b. Research Activities and Results

Objective #1 Compare the *in vitro* response of normal and dysplastic chondrocytes to endocrine, paracrine and autocrine factors thought to control the life cycle of epiphyseal growth plate chondrocytes.

Results A major focus of this research has been to investigate the role of growth hormone in chondrocyte physiology since this is the systemic hormone responsible for longitudinal bone growth. In contrast to mammalian chondrocytes, growth hormone was not mitogenic for avian growth plate chondrocytes maintained in cell culture. However, growth hormone was essential for chondrocyte survival in long-term culture and in combination with epidermal growth factor stimulated cell proliferation. Growth hormone treatment also results in time-dependent changes in extracellular matrix constituents: an increase in type II collagen gene expression accompanied by a decrease in the appearance of osteopontin in the medium. Cultured growth plate chondrocytes also express the receptor for growth

hormone which is capable of binding growth hormone. Thus, it is concluded that the inability of growth hormone to influence cell proliferation was not due to a lack of receptors, but appears to be due to events further downstream (1,2).

Fibroblast growth factor (bFGF) is a peptide growth factor which was found to be important for chondrocyte proliferation. Basic fibroblast growth factor was localized in the proliferative zone of the epiphyseal growth plate as well as chondrocytes isolated from this tissue. Although bFGF was present in these cells and extracellular matrix, it seemed to be unavailable for biological activity since exogenous bFGF was required for cell proliferation. The sequestered bFGF could be released to stimulate cell proliferation by treating cultures with the proteolytic enzyme plasmin. It was concluded that bFGF is synthesized by chondrocytes and functions as an autocrine/paracrine mitogen via its deposition in the extracellular matrix with subsequent release being the critical step for chondrocyte proliferation (3, 4, 5).

Objective #2 Determine the gene products of normal and dyschondroplastic chondrocytes with special emphasis on those EMC components specifically associated with cell hypertrophy and the formation of a calcified cartilage matrix.

Results The matrix proteins osteopontin, osteonectin, and type X collagen, as well as the enzyme alkaline phosphatase, are markers for chondrocyte hypertrophy. Although the expression of these gene products is low in rapidly proliferating chondrocytes, these genes are readily expressed when chondrocytes are induced to differentiate. This phenomenon is readily observed with chondrocytes isolated from normal growth plate tissue (6).

However, our ability to study gene expression in cells isolated from TD tissue has been impaired by a number of factors: (1) yield of viable cells is extremely low, (2) the cells are heterogeneous with respect to immunostaining for bFGF, osteonectin and osteopontin, (3) extraction of isolated cells yields quantities of mRNA insufficient for northern blot analysis.

Genomic DNA was prepared from chondrocytes isolated from the proliferative and hypertrophic zones of normal growth plate and TD lesions. Genomic DNA from cells of the TD lesion shows the nucleosomal laddering indicative of apoptosis, while proliferative and hypertrophic cells do not. This

result was confirmed by the use of the Cell Death Detection Assay[™] which shows quantitatively that cells from TD lesions contain significantly more nucleosomal fragments than cells from the proliferative or hypertrophic zones. *In situ* examination of the epiphyseal growth plate with terminal deoxynucleotidyl transferase mediated dUTP nick end labeling (TUNEL) clearly shows that the cells of the TD lesion are apoptotic. Small, medium and large lesions all contain apoptotic cells with larger lesions showing progressively more intense labeling by TUNEL (7). This observation is illustrated in figure 4.

We believe that these results explain the observations of our laboratory and others that a variety of genes show reduced expression within the TD lesion when examined by *in situ* hybridization. It is our opinion that examination of the cells comprising the lesion in order to establish the underlying cause of TD is futile in light of the apoptotic condition of these cells. We propose that future research into the cause of TD should focus on comparing gene expression in the non-apoptotic pre-hypertrophic zone immediately proximal to lesion with gene expression in the prehypertrophic zone of the normal growth plate.

Objective #3 Utilize histochemical and immunocytochemical techniques to study the formation and distribution of extracellular matrix components in normal and dysplastic tissue.

Results The use of histochemical, immunocytochemical and *in situ* hybridization techniques provided the greatest insight into the possible mechanisms involved in the development of tibial dyschondroplasia. Furthermore, these techniques were applied to normal and dysplastic tissue in order to compare the pattern of expression of the extracellular matrix components osteopontin, osteonectin and type X collagen. These extracellular matrix components are associated with chondrocyte hypertrophy and therefore were found to be localized in the hypertrophic zone of normal epiphyseal growth plates. Although these markers were found to be present in the upper hypertrophic zone above the lesion, they were not expressed within the lesion. An example is shown in figure 5. A similar pattern was observed with the enzyme alkaline phosphatase, an enzyme associated with normal hypertrophic chondrocytes. Similar patterns were observed in all tissues examined whether the lesions were spontaneous or induced by dietary treatments of genetic selection. These results lead to the conclusion that the growth

plate chondrocytes associated with the TD lesion start to differentiate but then de-differentiate so that they resemble chondrocytes in the pre-hypertrophic zone (8, 9, 10).

A similar pattern was also observed for fibroblast growth factor (bFGF). The peptide was of particular interest since it is the most potent angiogenic substance known. Since TD lesions are not vascularized in a normal manner, bFGF is a candidate for involvement in this process of cartilage vascularization. In the normal growth plate, bFGF is localized in both the proliferative and hypertrophic zones. As was observed with the extracellular matrix components, bFGF was present anterior and posterior to the lesion with greatly diminished immunological activity in the lesion proper. In contrast to the results described above, the expression of the growth hormone receptor was not perturbed in the TD tissue. This observation is of particular interest since previous research had indicated that chickens with TD had elevated levels of plasma growth hormone (11, 12).

Another significant observation relates to the apparent heterogeneity of the chondrocytes in the TD lesion. When TD tissue is stained with conventional histological stains such as H & E, the cells are homogeneous in appearance and staining properties. When the TD tissue is examined immunologically or by *in situ* hybridization, a few chondrocytes are positive with the majority of the cells being negative. Similar results were obtained with chondrocytes isolated from the TD lesion. Only a few of the isolated cells stained positive for bFGF or osteonectin. This is in contrast to normal growth plate tissue where chondrocytes within a given zone were uniformly positive for a given matrix marker or peptide such as bFGF (8, 9, 10).

DISCUSSION

Since previous research in several laboratories indicated that TD tissue lacked the markers for the final stages of chondrocyte differentiation, we hypothesized that TD was the result of a failure in the differentiation process. However, our results have demonstrated that the growth plate anterior to the TD lesion exhibits many of the markers of chondrocyte hypertrophy. Thus, it would appear that the lesion chondrocytes de-differentiate, losing these markers of hypertrophy in the process. Once these chondrocytes start to de-differentiate, many of these cells undergo apoptosis. It is entirely possible that

many of these changes are the result of the TD lesion rather than the initial cause of the development of the lesion.

We propose that future research into the metabolic cause of TD should focus on gene expression in the non-apoptotic pre-hypertrophic zone immediately proximal to TD lesion with emphasis on endocrine and autocrine factors which cause chondrocytes to de-differentiate. This approach should provide insight into the metabolic defect involved in the development of this important skeletal disorder.

DESCRIPTION OF COOPERATION: Investigators at Penn State have been responsible for supplying most of the tissues for *in situ* hybridization and immunocytochemistry. The genetic lines have been extremely valuable as a consistently reliable source of desired tissue (TD vs. normal). Investigators from Israel have provided reagents for the immunocytochemistry and performed all of the *in situ* hybridization procedures. Both laboratories have performed experiments in the *in vitro* culture of growth plate chondrocytes.

CONCLUSIONS: We feel that this has been an extremely valuable collaboration. Both laboratories were able to focus their expertise towards the solution of a common problem. The hypothesis that tibial dyschondroplasia is the result of the failure of chondrocytes to differentiate was thoroughly tested. It is now clear that this is not the explanation but rather that chondrocytes start to differentiate but fail to complete this process.

These results suggest that future research should focus on the earliest detectable stages in the development of TD and examine endocrine and autocrine factors which cause chondrocytes to differentiate. The final step would be the identification of nutritional and management protocols which impact on the mediators of chondrocyte differentiation.

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Figure 1. Longitudinal section of the proximal end of the tibia of a 4-week-old broiler chicken. Bone on left exhibits normal morphology. Bone on the right has a severe tibial dyschondroplastic lesion. This lesion is characterized by a plug of opaque cartilage associated with the growth plate and extending into the metaphysis.

Figure 2. Light micrograph of sections of normal and dysplastic tissue stained with alcian blue-PAS. Section on left is taken from the hypertrophic zone of a normal growth plate and shows the tip of an invading metaphyseal blood vessel. Section on the right is from the proximal region of a dysplastic lesion. Note differences in staining properties of the cells and extracellular matrix. (x 480).

FIGURE 3

PATTERN OF GENE PRODUCTS IN AVIAN EPIPHYSEAL GROWTH PLATE

M = METAPHYSEAL VESSEL

Modified from Poole (1991) and Howlett (1980)

FIGURE 4. Transmission micrographs of the proliferative-hypertrophic zones (A) of the epiphyseal growth plate and of the TD lesion (B). Fluorescence micrographs of A & B labeled with TUNEL, demonstrate that the cells of the TD lesion (D) are apoptotic while little staining is observed in the proliferative-hypertrophic zones (C).

FIGURE 5. H & E Staining of normal epiphyseal growth plate (1) and TD lesion (2). Dark field *in situ* hybridization with avian osteopontin gene in normal (A) and TD tissue (B).